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Identification and molecular characterization of new STLV-1 and STLV-3 strains in wild-caught nonhuman primates in Cameroon

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Abstract

Humans and simian species are infected by deltaretroviruses (HTLV and STLV respectively), which are collectively called primate T-cell lymphotropic viruses (PTLVs). In humans, four types of HTLV have been described (HTLV-1 to -4) with three of them having closely related simian virus analogues named STLV-1, 2 and 3. In this study, our aim was to search for a simian HTLV-4-related virus and to document and characterize further the diversity of STLV infections in wild primate populations. We screened 1297 whole blood samples from 13 different primate species from southern Cameroon. Overall, 93 samples gave HTLV-1, HTLV-2 or dual HTLV-1/-2 INNOLIA profiles, 12 were HTLV positive but untypeable and 14 were indeterminate. Subsequently, we performed generic and specific (STLV-1 to -3) *tax-rex* PCRs to discriminate the different PTLV types, completed with phylogenetic analysis of 450-bp LTR sequences for STLV-1 and 900 bp pX-LTR sequences for STLV-3. We show for the first time that *Lophocebus albigena* and *Cercopithecus cephus* carry both STLV-1 and a divergent STLV-3. We also identified a new STLV-1 lineage in one *C. cephus*. Finally, we identify relative divergence levels in the *tax/rex* phylogeny suggesting that additional types of PTLV should be defined, particularly for the highly divergent STLV-1(MarB43) strain that we provisionally name STLV-5.

Keywords: PTLVs; STLV-1; STLV-3; Nonhuman primate; Cross-species transmission; Phylogeny; Virus–host species co-evolution; Cameroon

Introduction

Primate T-lymphotropic viruses (PTLVs) include both human and simian T-cell lymphotropic viruses (HTLVs and STLVs, respectively). To date four types, HTLV type 1 to 4, have been described in humans with three of them having a simian counterpart, named STLV 1, 2 and 3, respectively (Courgnaud et al., 2004; Giri et al., 1994; Goubau et al., 1994; Koralnik et al., 1994; Van Dooren et al., 2001a,b). A simian virus analogue to the recently discovered HTLV-4 has yet to be identified (Wolfe et al., 2005). Similar to human immunodeficiency viruses (HIVs), HTLV types 1 and 2 have also spread widely into human populations in most parts of the world. While HTLV-1 has spread worldwide, HTLV-2 is mainly restricted to central Africa, Amerindians in South America, and some IDU (intravenous drug user) populations in Europe and the US. HTLV-1 causes adult T-cell leukemia, neurological disorders such as HTLV-1-associate myelopathy (HAM) also known as tropical spastic paraparesis (TSP), and has also been associated with inflammatory diseases (Gessain et al., 1985; Osame et al., 1987). HTLV-2 is less pathogenic than HTLV-1 and only few cases of a neurological disease similar to HAM/TSP have been documented (Koralnik et al., 1994; Rosenblatt et al., 1986; Sheremata et al., 1993). Very limited information is available to assess disease associations with

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the newly found HTLV-3 and HTLV-4 in Cameroonian nonhuman primate (NHP) hunters (Calattini et al., 2006, 2005; Switzer et al., 2006; Wolfe et al., 2005). Nevertheless, the full-length sequence analysis of the HTLV-3 genome suggests a pathogenic potential in HTLV-3-infected individuals similar to that observed in HTLV-1 infection (Switzer et al., 2006). Only partial sequences are currently available from the single HTLV-4 infected person limiting evaluation of genetic markers of pathogenesis for this virus (Wolfe et al., 2005).

Phylogenetic analysis of PTLV sequences suggests that HTLV infection most likely originated from multiple cross-species transmissions from STLV-infected NHPs (Calattini et al., 2005; Gessain and de The, 1996; Koralnik et al., 1994; Liu et al., 1996; Nerrienet et al., 2001; Slattery et al., 1999; Switzer et al., 2006; Van Dooren et al., 2001b; Vandamme et al., 1998; Wolfe et al., 2005). Cross-species transmissions between different NHPs have also been documented suggesting the ease at which STLV crosses species barriers (Georges-Courbot et al., 1996; Makuwa et al., 2004b; Nerrienet et al., 1998). STLVs have been isolated from a broad variety of Old World monkeys. STLV-1 is widely distributed in Asian and African NHPs including *Cercopithecidae* (*Cercopithecinae* and *Colobinae*) and *Hominoidae* primate families and cannot be separated into distinct phylogenetic lineages according to their host origin but are rather separated by their geographic origin (Vandamme et al., 1998). In contrast, STLV-2 has only been identified in captive bonobos (*Pan paniscus*) from the Democratic Republic of Congo (Giri et al., 1994; Vandamme et al., 1996). STLV-3 has been found in several African NHPs, including *Papio hamadryas*, *P. anubis* and *Theropithecus gelada* in East Africa and *Cercocebus torquatus*, *Cercocebus agilis*, *P. papio* and *Cercopithecus nictitans* in West-Central Africa. Different STLV-3 subtypes have been proposed according to the geographic origin of the host species (Courgnaud et al., 2004; Goubau et al., 1994; Meertens and Gessain, 2003; Meertens et al., 2002, 2003; Takemura et al., 2002; Van Brussel et al., 1996; Van Dooren et al., 2001a,b, 2004). Interestingly, both STLV-1 and STLV-3 infections have been observed in *C. nictitans* and *C. agilis*, and STLV-1 and STLV-3 co-infections have even been described in *C. agilis* species in Cameroon as well as in wild baboons from Ethiopia (Courgnaud et al., 2004; Takemura et al., 2002).

Here we investigate the diversity of STLV infection in wild primate populations from Cameroon to better understand the possible simian origin of HTLV-4 identified recently in a Cameroonian hunter and to better understand the zoonotic origin of other HTLVs.

Results

Study population and serologic screening for STLV

Whole blood was obtained from 1297 NHPs, representing 13 different species. For 151 samples, species identification had not been done in the field (Table 1). Wild-caught animals (bushmeat monkeys) were sampled in villages and nearby logging concessions from different areas in southeastern and southwestern Cameroon as depicted in Fig. 1. Fourteen samples were collected from monkeys kept as pet animals, all of which were juveniles. In order to detect PTLV infection in NHPs, we used commercially available HTLV-1/HTLV-2 assays since all previously reported STLV infections were also identified through cross-reactivity with HTLV antigens. A total of 169 samples (13%) reacted in the HTLV-1/HTLV-2 ELISA (ratio OD/cut-off ≥ 1). All ELISA reactive samples were re-tested with the INNOLIA HTLV I/II confirmatory assay and the results are summarized in Table 1. A total of 119 specimens

Table 1
Detection of HTLV-1 and HTLV-2 cross-reactive antibodies in wild primate species in Cameroon

Species	Common name	No. of animals tested	No. of ELISA reactive samples [a]	Results of the INNOLIA-HTLV-1/2 confirmatory assay				
				HTLV-1	HTLV-2	HTLV-1+2	Indeterminate and untypeable HTLV [b]	HTLV negative
Cercopithecus pogonias	Crested mona	202	26	12 [c]	0	0	3	9
Cercopithecus neglectus	de Brazza's monkey	4	0	0	0	0	0	–
Cercopithecus cephus	Mustached monkey	411	28	4	2	1	6	15
Cercopithecus nictitans	Greater spot-nosed monkey	307	24	5	0	0	4	15
Miopithecus ogouensis	Gabon talapoin	8	3	3	0	0	0	–
Cercocebus torquatus	Red-capped mangabey	2	1	0	1	0	0	–
Cercocebus agilis	Agile mangabey	81	51	35	5	3	5	3
Lophocebus albigena	Grey-cheeked mangabey	103	22	1	11	1	7	2
Mandrillus sphinx	Mandrill	7	2	2	0	0	0	–
Colobus satanus	Black colobus	8	0	0	0	0	0	–
Colobus guereza	Mantled guereza	7	3	0	0	0	0	3
Pan troglodytes	Chimpanzee	5	0	0	0	0	0	–
Gorilla gorilla	Gorilla	1	0	0	0	0	0	–
Species not determined		151	9	6	1	0	1	1
Total (%)		1297	169 (13%)	68 (5.3%)	20 (1.5%)	5 (0.4%)	26 (2.0%)	48 [d]

[a] Samples were considered as ELISA reactive when the OD/CO ratio ≥ 1.
[b] Includes 12 untypeable and 14 indeterminate samples.
[c] Number of samples observed.
[d] Sufficient biological material from two samples was not available for INNOLIA testing.

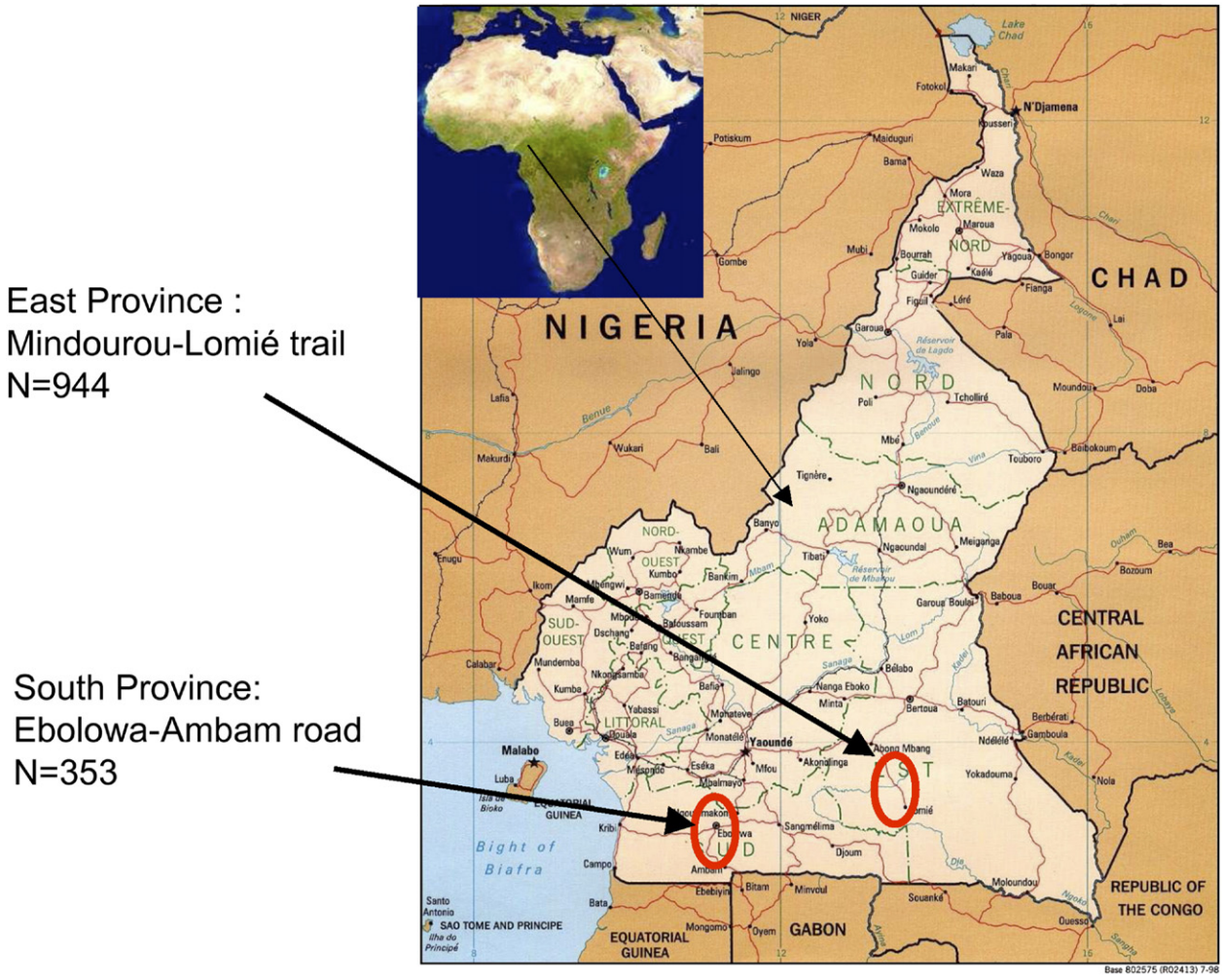


Fig. 1. Nonhuman primate sampling sites in southern Cameroon.

were seroreactive in the INNOLIA assay. A variety of INNOLIA profiles were observed ranging from HTLV-1-like ($n=68$; 5.3%), HTLV-2-like ($n=20$; 1.5%), dual HTLV-1/-2-like ($n=5$; 0.4%), HTLV positive but untypeable ($n=12$; 0.9%), to indeterminate ($n=14$; 1.1%). Forty-eight specimens tested negative in the INNOLIA test. Sufficient biological material from two samples was not available for INNOLIA testing. Overall, positive HTLV cross-reactive antibodies were detected in 8 out of 13 NHP species and in 9 out of 151 samples for which species identification was not available. The overall STLV seroprevalence in our primate bushmeat samples, excluding seroindeterminates, was 8.1% (105/1297). None of the samples collected among pet monkeys was seroreactive.

Species identification was conducted on site by visual inspection according to the primate taxnomical classification provided in the *Kingdon Field Guide to African Mammals* (Kingdon, 1997). Nevertheless, in order to verify the primate species identification done in the field, we analyzed the mt 12S rRNA gene and/or the glucose-6-phosphate dehydrogenase genes as previously described on all samples with HTLV-2-like, HTLV-1+2-like, HTLV indeterminate (including positive HTLV but untypeable) and some HTLV negative and some HTLV-1-like INNOLIA profiles (Peeters et al., 2002; van der Kuyl et al., 2000). Our results indicated that about 14% of monkey species were initially misidentified (data not shown), probably as a result of some animals being dead for more than 24 h.

Confirmation of STLV infection by confirmatory and discriminatory PCR analyses of the tax gene

In order to confirm whether animals with HTLV-like antibodies were infected with a PTLV and to determine the type of PTLV, we performed PCR using highly generic *tax* primer pairs previously shown to detect a large diversity of HTLV and STLV strains, and known to have a high sensitivity and specificity in characterizing the PTLV type (Vandamme et al., 1997). Among the 167 samples with a positive, indeterminate or negative HTLV-like INNOLIA profile, a subset of 103 specimens were selected for PCR testing according to the species, the sampling site, as well as the cross-reactivity with the ELISA tests and the INNOLIA results (for example, weakly reactive ELISA results with negative INNOLIA profiles). These 103 samples were then tested by generic PCR followed by type-specific PCR to discriminate between STLV-1, STLV-2 and STLV-3 (Table 2). Among the 103

Table 2
STLV serological profiles and genotypes present in wild nonhuman primates from Cameroon

Species	INNOLIA HTLVI/II results	No. of samples tested	No. of samples positive for tax sequences for			No. of samples negative for tax sequences
			STLV-1	STLV-3	STLV-1+3	
Cercopithecus cephus	HTLV-1	4	4	0	0	0
	HTLV-2	2	0	1	0	1
	HTLV-1+2	1	1	0	0	0
	Indeterminate HTLV	6	2	1	0	3
	Negative	14	0	0	0	14
Cercopithecus nictitans	HTLV-1	2	2	0	0	0
	Indeterminate HTLV	4	1	2	0	1
	Negative	15	0	0	0	15
Cercopithecus pogonias	HTLV-1	1	1	0	0	0
	Indeterminate HTLV	5	2	0	0	3
	Negative	8	0	0	0	8
Lophocebus albigena	HTLV-1	1	1	0	0	0
	HTLV-2	9	0	9	0	0
	HTLV-1+2	1	0	1	0	0
	Indeterminate HTLV	7	0	6	0	1
	Negative	2	0	0	0	2
Cercocebus agilis	HTLV-1	2	2	0	0	0
	HTLV-2	5	0	4	1	0
	HTLV-1+2	3	2	0	1	0
	Indeterminate HTLV	5	1	3	0	1
	Negative	3	0	0	0	3
Cercocebus torquatus	HTLV-2	1	0	1	0	0
Colobus guereza	Negative	2	0	0	0	2
Subtotal	HTLV-1	10	10	0	0	0
	HTLV-2	17	0	15	1	1
	HTLV-1+2	5	3	1	1	0
	Indeterminate HTLV	27	6	12	0	9
	Negative	44	0	0	0	44
Total (%)		103	19 (18.5)	28 (27.2)	2 (1.9)	54 (52.4)

samples, 10 were positive for HTLV-1-like antibodies, 17 were positive for HTLV-2-like antibodies, 5 gave dual HTLV-1/-2 profiles, 27 were indeterminate and 44 were negative in the INNOLIA HTLV-1/2 assay. All 10 HTLV-1-like samples were reactive with the STLV-1 specific *tax* primers only. Among the 17 HTLV-2-like samples, 15 were reactive with the STLV-3 specific *tax* primers only, 1 was reactive with both STLV-1 and STLV-3 specific *tax* primers and 1 was negative by generic PCR. For the 5 samples with HTLV-1/-2 dual profiles, 3 were reactive with the STLV-1 specific *tax* primers only, 1 with the STLV-3 specific *tax* primers only and 1 was positive with both STLV-1 and STLV-3 specific *tax* primers. Among the 26 indeterminate samples, 4 were reactive with the STLV-1 specific *tax* primers, 12 were reactive with the STLV-3 specific *tax* primers and 10 could not be amplified at all, not even with generic primers. All 44 ELISA reactive but INNOLIA negative samples were negative by generic and type specific PCR. The *tax* PCR results are summarized in Table 2. Overall, these results confirm previous observations of STLV-1 and -3 infections in the sampled primate species and show for the first time that *Lophocebus albigena* (grey-cheeked mangabeys) and *Cercopithecus cephus* (mustached monkey) carry both STLV-1 and STLV-3.

All PCR results were further confirmed by sequence and phylogenetic analysis. The phylogenetic relationships of the 220-bp *tax/rex* sequences from the STLVs isolated in this study are shown in Fig. 2. All STLV-1 strains fell in the cluster of PTLV-1 strains whereas STLV-3 strains clustered with PTLV-3 sequences. The newly characterized STLV-3 strains from *L. albigena* and *C. cephus* grouped together but were distinct from other PTLV-3 strains (Fig. 2). To ensure that this latter result did not result from a laboratory contamination, new DNA extractions and *tax/rex* PCRs for the *C. cephus* samples were performed and confirmed our initial findings. Interestingly, all PTLV-3-like sequences from Cameroonian *C. nictitans* (greater-spot nosed monkey) formed a monophyletic clade highly divergent from all known PTLV-3s. The phylogenetic relationships of these new viruses within either STLV-1 or STLV-3 lineages were further analyzed based on LTR (STLV-1) and *tax*-pX-LTR (STLV-3) fragments sequence comparison as described below.

Analysis of STLV-1 LTR sequences

In order to confirm and study more in detail the phylogenetic relationships observed in the *tax-rex* region, a 450-bp fragment of the LTR region was sequenced for 7 STLV-1-infected animals: three *C. nictitans*, two *C. cephus*, one *C. pogonias* (crested mona) and one *L. albigena*. As for the phylogeny of the *tax/rex* region, all new STLV-1 LTR sequences clustered with the African PTLV-1 strains and more precisely with the HTLV-1 subtype D and F (Fig. 3). Among the HTLV-1 subtype D clade, new STLV-1 strains from *C. pogonias*, *C. cephus* and *C. nictitans* were closely related to one another and with the previously described STLV-1 sequences from a Cameroonian crested mona,

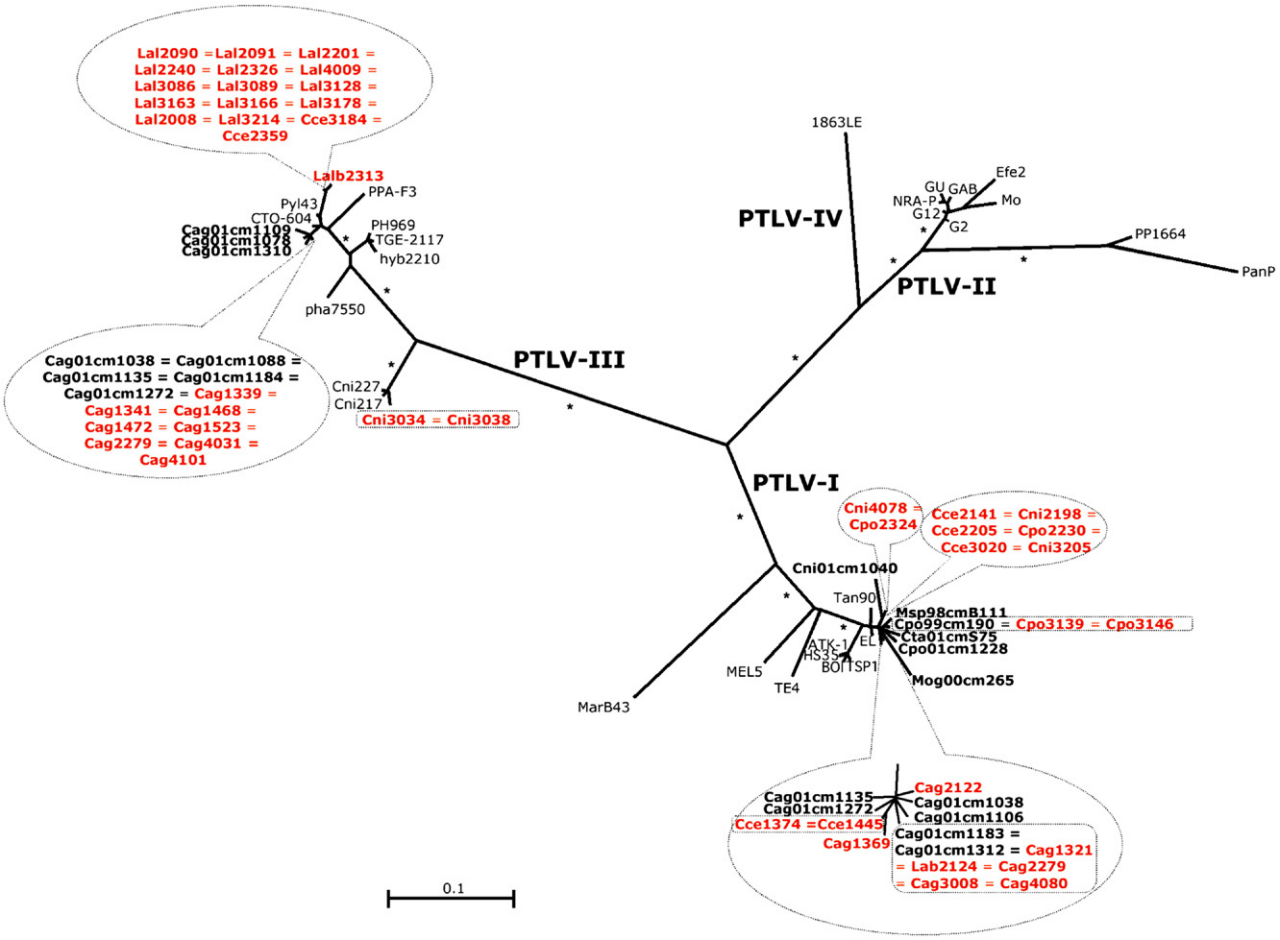


Fig. 2. PTLV phylogeny inferred using 220-bp tax-rex sequences. Numbers correspond to internal branch support derived from 500 bootstrap replicates (only values above 70% are shown). Scale bar represents the number of nucleotide substitutions per site. Groves' primate taxonomy nomenclature is used (Groves, 2001). Nonhuman primates are coded using the first letter of the genus followed by the first two letters of the species name: *Cercopithecus nictitans*=Cni, *Lophocebus albigena*=Lal, *Cercocebus agilis*=Cag, *Cercopithecus cephus*=Cce, *Cercopithecus pogonias*=Cpo. Sequences in red and bold text are from the current and a previous study, respectively (Courgnaud et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a greater-spot nosed monkey from Gabon and mandrills (Courgnaud et al., 2004; Makuwa et al., 2004a,b). The new STLV-1 strains from *L. albigena* and *C. nictitans* clustered within the HTLV-1 subtype F clade and exhibited a high level of sequence identity (98 to 99%) with the previously published *C. agilis* and *C. cephus* STLV-1 sequences (Courgnaud et al., 2004). Interestingly, one *C. cephus* STLV-1 strain (01CM1374) did not cluster with the previously known *C. cephus* STLV-1 strains (01CM2124 in HTLV-1 subtype D and 01CM2474 in HTLV-1 subtype F) and did not group at a significant bootstrap level within any STLV-1 lineage (Fig. 3) suggesting identification of a new lineage. Support for this potential new STLV-1 lineage and further resolution of this lineage from the closest sister phyla, i.e. the two monophyletic lineages within the subtype F clade inferred in the PTLV-1 LTR phylogeny (Fig. 3), was evaluated using the four-cluster likelihood mapping method. This method has been previously used to define the novel HTLV-1 E and -1 F subtypes (Salemi et al., 1998). The four sequence subsets considered were as follows: (a) 01CM1374; (b) all STLV-1 strains in the HTLV-1 F cluster comprising *C. agilis*, *C. cephus*, *C. nictitans* and *L. albigena*; (c) HTLV-1 F(Lib2) and mandrill STLV-1(mnd9); (d) all other strains in the phylogeny. The triangle in which the tree likelihoods are plotted can be divided into regions of support for each of the three possible topologies, with unresolved trees appearing in the center (Fig. 4). Just 2.4% of the quartets supported the clustering of 01CM1374 with the *C. agilis* (agile mangabeys), *C. cephus*, *C. nictitans* and *L. albigena* sequences in the HTLV-1 subtype F clade, whereas the remaining quartets (97.6%) corresponded to an unresolved tree. These results are consistent with the 01CM1374 LTR sequence representing a new lineage among PTLV-1.

Phylogenetic analysis of STLV-3 tax-pX-LTR sequences

Similarly as for STLV-1 positive samples, we studied also an additional genomic region for a subset of STLV-3 positive samples in order to confirm and study more in detail the phylogenetic relationships observed in the *tax-rex* region. A 900-bp fragment spanning the *tax-rex* and pX-LTR region was sequenced from eight STLV-3-infected animals consisting of four *L. albigena*

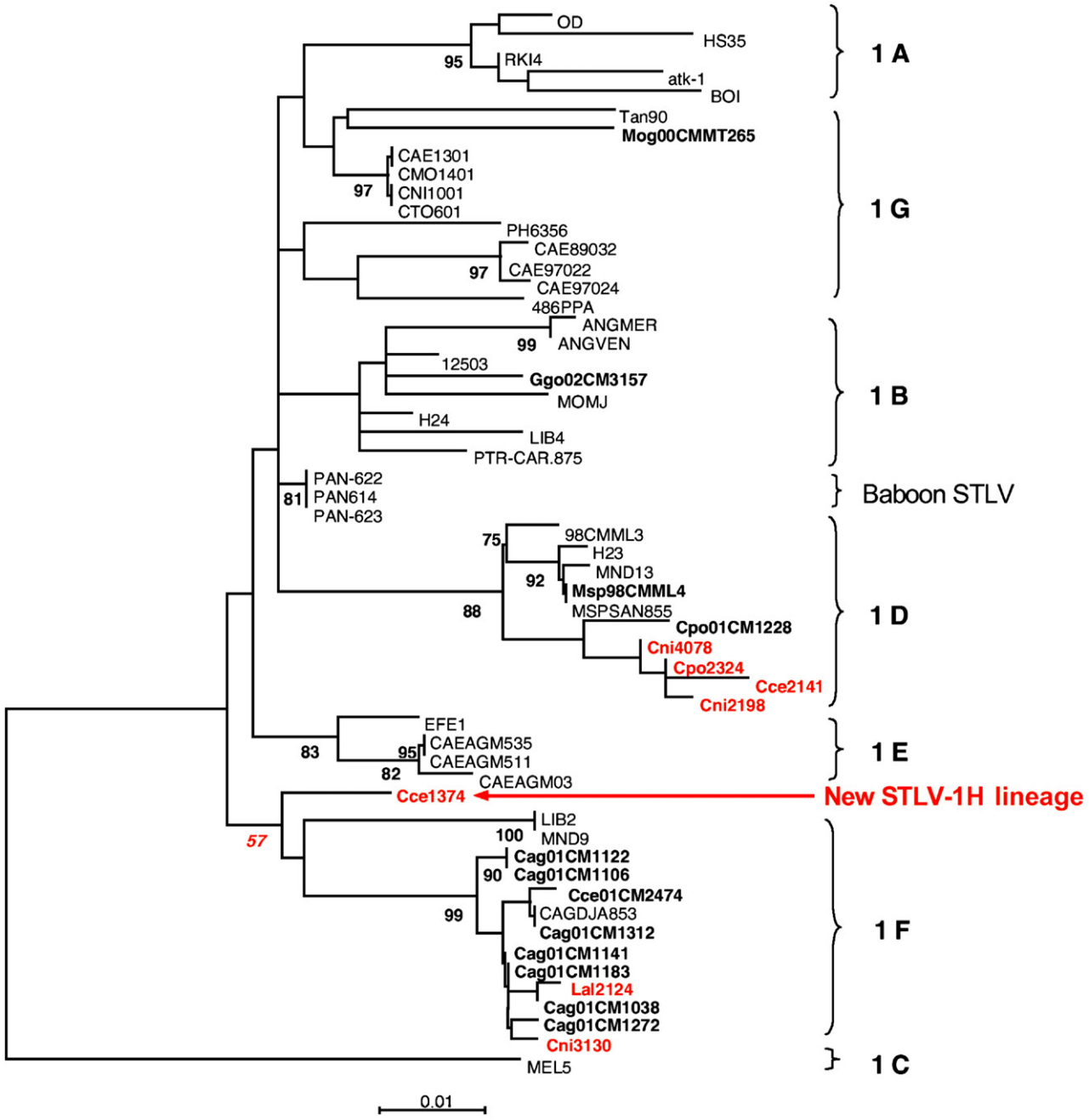


Fig. 3. Inference of PTLV-1 phylogeny using 450-bp LTR sequences. Numbers correspond to internal branch support derived from 500 bootstrap replicates (only values above 70% are shown except for the non-significant bootstrap percentage (<70%) for the internal branch grouping Cce01CM1374 to HTLV-IF). Scale bar represents the number of nucleotide substitution per site. Groves' primate taxonomy nomenclature is used (Groves, 2001). Nonhuman primates are coded using the first letter of the genus followed by the first two letters of the species name: *Cercopithecus nictitans*=Cni, *Lophocebus albigena*=Lal, *Cercocebus agilis*=Cag, *Cercopithecus cephus*=Cce, *Cercopithecus pogonias*=Cpo. Sequences in red and bold text are from the current and a previous study, respectively (Courgnaud et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampled at four distinct sites, one *C. cephus* and three *C. agilis* (Fig. 5). We failed to amplify STLV-3 *tax*-pX-LTR sequences from seroreactive *C. nictitans* samples probably because of the high genetic diversity of these viruses. The new STLV-3 sequences from agile mangabeys were closely related to previously described STLV-3 sequences obtained from *C. agilis* captured in southern Cameroon (Courgnaud et al., 2004), whereas STLV-3 from the grey-cheeked mangabeys formed a separate monophyletic lineage (Fig. 5). Surprisingly, the STLV-3 from the *C. cephus* grouped within the *L. albigena* STLV-3 cluster (Fig. 5). Accordingly, *tax*-pX-LTR from the *C. cephus* and *L. albigena* STLVs exhibited a very high level of sequence identity (99 to 100%).

Comparison of genetic diversity between PTLV lineages

In order to assess the genetic relationships between the various PTLV lineages, we further explored their relative levels

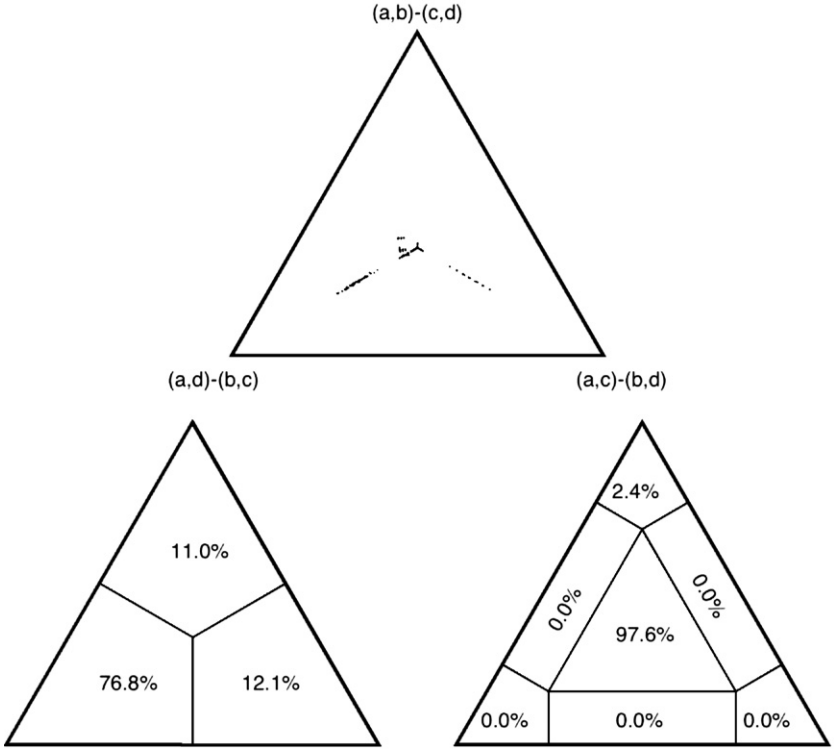


Fig. 4. Four-cluster likelihood mapping analysis for STLV-1 (Cce 01CM1374) of all possible quartets (924) using LTR sequences. Numbers in each corner of the lower triangles represent the percentage of quartets for which a particular topology is supported. In the lower right triangle, the number in the center triangle represents the percentage of quartets associated with a star-like phylogeny. The letters a–d represent the subsets of strains as follows: (a) 01CM1374; (b) subtype 1F STLV-only strains; (c) subtype 1F HTLV (Lib2) and mandrill STLV (mnd9) strains; (d) all other strains in the phylogenetic tree presented in Fig. 3. The letters between parentheses indicate subsets clustering together.

of sequence divergence in the *tax/rex* phylogeny using the TreePAT package in the TreeDyn program (Chevenet et al., 2006). TreePAT allows the visualization of patterns of genetic relationships described by a phylogenetic tree, in the form of a colored matrix. A pairwise distance matrix is constructed by summing the branch lengths in the tree between each pair of taxa. The distances thus calculated range from 0 (a taxon to itself) to the longer distance between two taxa. Distance classes can be empirically defined by setting an upper distance value. The pairwise distances within a given class appear in the matrix as colored squares, thus permitting the visual comparison of taxa levels of divergence. Using the *tax/rex* phylogeny, we varied the genetic distance ranges to delineate phylogroups consistent with the accepted PTLV classification as well as with monophyletic clades obtained in our phylogenetic analyses (Fig. 6). For this analysis, we were more interested in the taxonomic status of the most divergent lineages within each of the major PTLV divisions. Using this approach, we identified three main patterns of PTLV diversity based on substitutions per site in the branch lengths of the inferred phylogenetic relationships: (i) for the distance class 0–0.419 substitutions per site, only three major genetic partitions were found: PTLV-1, PTLV-3 and the grouped PTLV-2 and -4 lineages (Fig. 6a); (ii) at the distance class 0–0.238 substitutions per site, the highly divergent STLV-1MarB43 lineage then separated from all other PTLV-I and the PTLV-2 and PTLV-4 sequences became distinct from each other (Fig. 6b); and (iii) further PTLV resolution was observed at the distance class 0–0.148 substitutions per site, with the *C. nictitans* PTLV-3-like sequences splitting from the PTLV-3 clade and the STLV-2 sequences (PanP and PP1664) becoming differentiated from the HTLV-2 cluster (Fig. 6c).

Discussion

Primary zoonotic HTLV infections most likely arise from the cross-species transmission of STLVs from infected NHPs through the hunting and butchering of primates for food, through injuries caused by pet primates like bites or scratches, or by close contact with NHP urine, feces or other body fluids. These primordial behaviors have exposed hunters over millennia to primate viruses and indeed phylogenetic analyses have inferred an ancient origin of HTLV in Africa (Salemi et al., 2000). Therefore, to better understand the zoonotic origin of HTLVs in Africa and to identify a putative simian counterpart to the recently discovered HTLV type 4 found in a Cameroonian hunter (Wolfe et al., 2005), we investigated the diversity of STLVs in hunted and pet primates in Cameroon. Using a combined approach of serological and PCR testing, we screened 1297 NHP samples mainly from moustached monkeys ($n=411$), greater spot-nosed monkeys ($n=307$), crested mona monkeys ($n=202$) and grey-cheeked mangabeys ($n=103$) from southern Cameroon. These primate species are commonly hunted for bushmeat in this country. The majority of these animals were sampled in southeastern Cameroon surrounding the geographic area where the HTLV-4-infected person was recently identified. Interestingly, some NHP specimens pre-

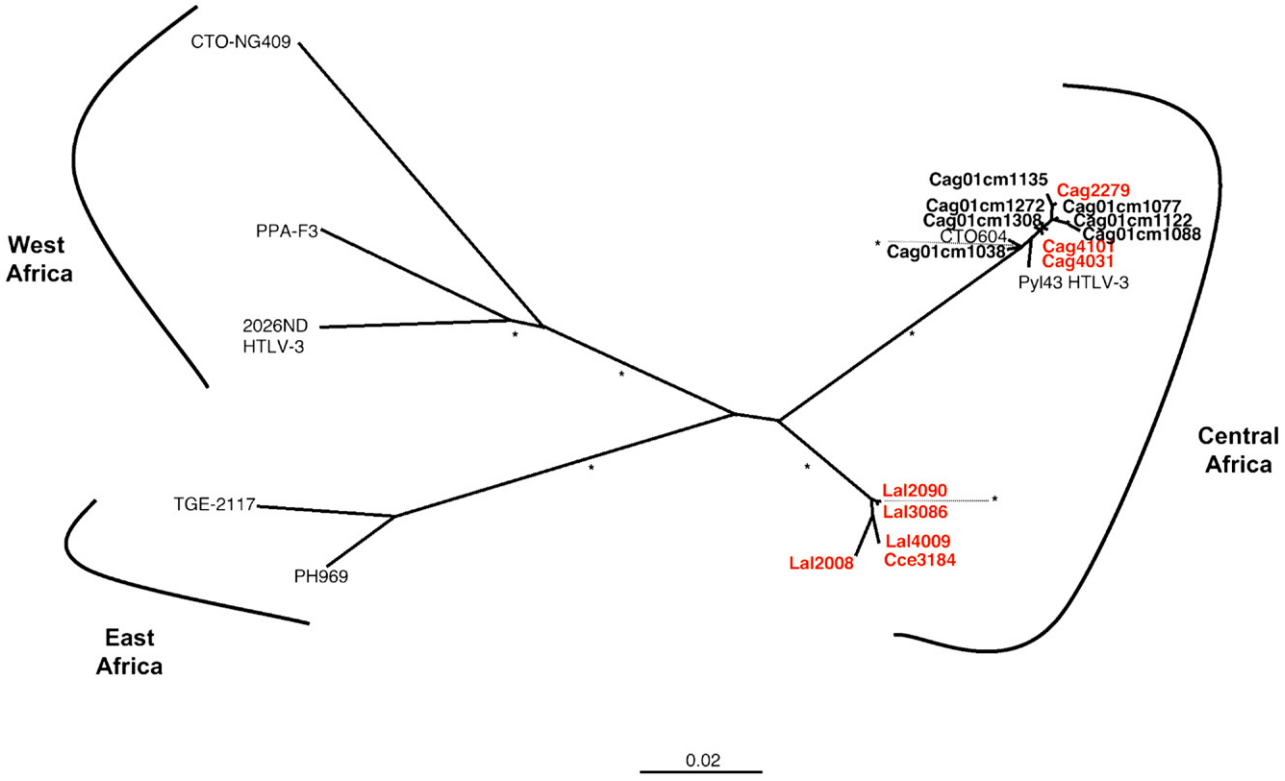
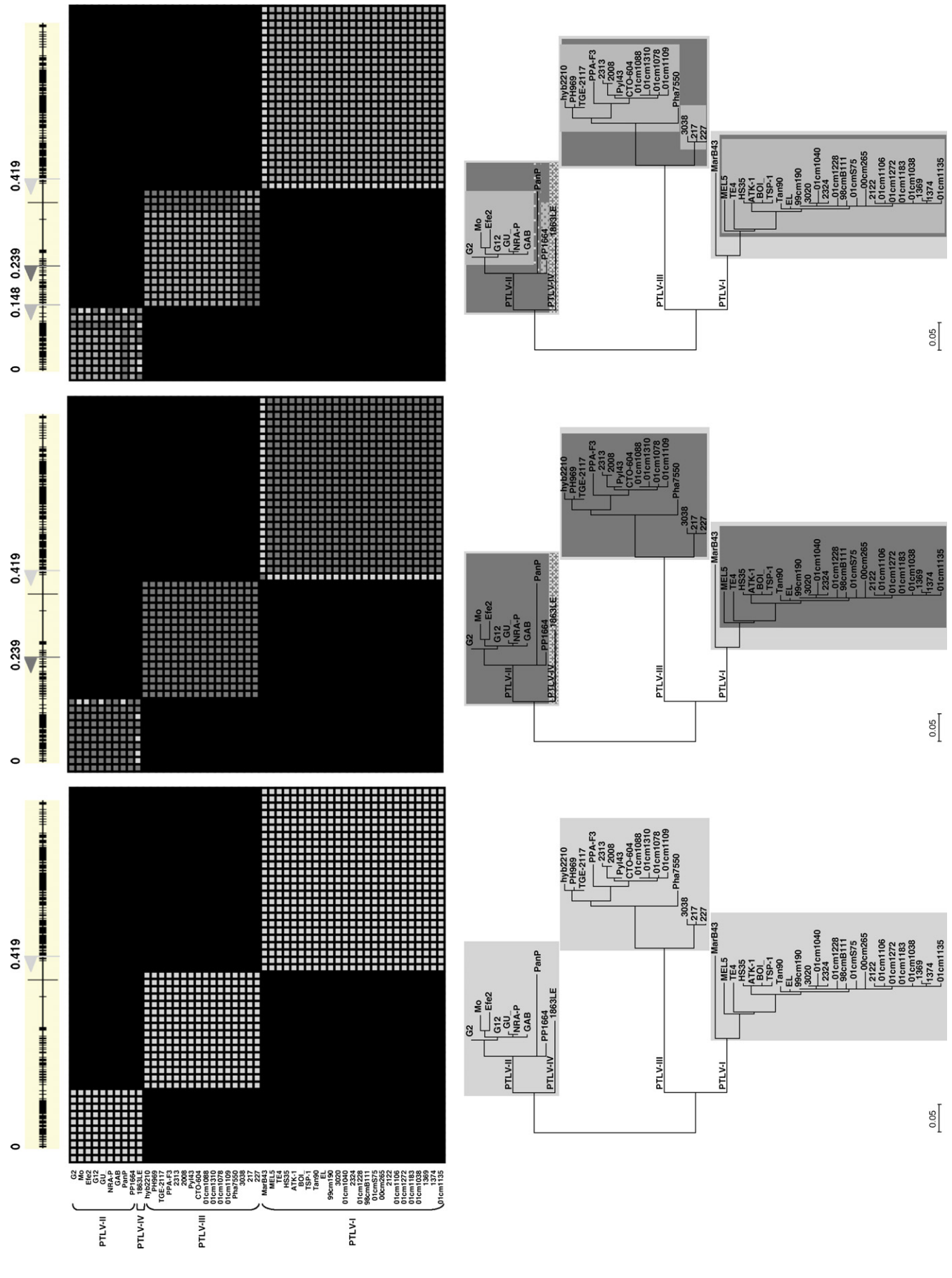


Fig. 5. Inference of PTLV-3 phylogeny using 900-bp tax-pX-LTR sequences. Numbers correspond to internal branch support derived from 500 bootstrap replications (only values above 70% are shown). Scale bar represents the number of nucleotide substitution per site. Primate taxonomy nomenclature is according to Groves (2001). Nonhuman primates are coded using the first letter of the genus followed by the first two letters of the species name: *Lophocebus albigena*=Lal, *Cercocebus agilis*=Cag, *Cercopithecus cephus*=Cce. Sequences in red and bold text are from the current and a previous study, respectively (Courgnaud et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sented HTLV-2 WB profiles like that observed in the HTLV-4-infected person (Wolfe et al., 2005). However, none of the NHP bushmeat or pet monkey samples we investigated contained PTLV sequences genetically related to HTLV-4. Nevertheless, to date, HTLV-4 is represented only by a single strain obtained from one person and could be rare in the human population as well as in NHPs (Wolfe et al., 2005). Population-based surveillance studies of humans and NHPs in central Africa are needed to determine the prevalence of HTLV-4 and to identify the simian counterpart of this novel virus, respectively.

It has been widely demonstrated that human populations in Central Africa are highly exposed to simian retroviruses (Courgnaud et al., 2004; Hahn et al., 2000; Hirsh et al., 1989; Peeters et al., 2002, 1989; Switzer et al., 2006; Vandamme et al., 1998; Wolfe et al., 2004). In the course of this study, we identified new STLV-1 and STLV-3 co-infections in *C. cephus* and *L. albigena* as well as new STLV-1 strains from both *C. cephus* and *C. nictitans*. These monkeys are commonly hunted for food in southern Cameroon placing hunters at increased risk for infection with these viruses. Phylogenetic analyses showed that these new STLV-1 strains are closely related to the PTLV-1 strains isolated in Central Africa and more precisely to the HTLV-1 subtypes D and F. More interestingly, we observed the co-circulation of different PTLV-1 subtypes within the same monkey species (*C. cephus* and *C. nictitans*) from a similar restricted geographic area. These results are consistent with the occurrence of multiple cross-species transmissions between primates and humans and also between different NHP species (Calattini et al., 2005; Koralnik et al., 1994; Switzer et al., 2006; Van Dooren et al., 2001b; Vandamme et al., 1998; Wolfe et al., 2005). Interestingly, one of the STLV-1 strains isolated from *C. cephus* monkey (O1CM1374) was equally divergent from STLV-1 from other *C. cephus* from Cameroon (O1CM2141 and 01CM2474) and did not cluster within any of the HTLV-1 subtypes from Central Africa. These results, combined with those from likelihood mapping analysis, suggest that strain O1CM1374 is the first member of a new African STLV-1 lineage that we name STLV-1 H.

Fig. 6. Relative levels of sequence divergence in the *tax/rex* phylogeny of PTLV lineages. Classes of distances identifying equivalent taxonomic divisions are colored in grey scale in the tree distance matrix (upper part of the figure). The upper limits of the distance classes retained in the analysis are positioned along a scale of all pairwise distances in the phylogeny (on top of the matrices). The relevant classes are as follows: (a) 0–0.419 substitution per site corresponding to the deeper level of divergence; (b) 0–0.239 substitution per site, superimposed onto the 0–0.419 class; (c) 0–0.148 substitution per site, superimposed onto 0–0.419 and 0–0.239 classes. Corresponding lineages are color-coded accordingly in the phylogenetic tree (lower part of the figures). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


0
0.419
0.148
0.239
PTLV-II
PTLV-IV
PTLV-III
PTLV-I
G2
Mo
Efe2
G12
GU
NRA-P
GAB
PanP
PP1664
1863LE
hyb2210
PH969
TGE-2117
PPA-F3
2313
2008
Pyl43
CTO-604
01cm1088
01cm1310
01cm1078
01cm1109
Pha7550
3038
217
227
MarB43
MEL5
TE4
HS35
ATK-1
BOI_1
TSP-1
Tan90
EL
99cm190
3020
01cm1040
2324
01cm1228
98cmB111
01cmS75
00cm265
2122
01cm1106
01cm1272
01cm1183
01cm1038
1369
1374
01cm1135
0.05

We also found a broad diversity of novel STLV-3s in hunted primates by analysis of *tax*-pX-LTR sequences from *L. albigena*, *C. cephus* and *C. agilis*. *C. agilis* STLV-3 were closely related to one another and formed a monophyletic clade with the previously described strains from wild-caught *C. agilis* and *C. torquatus* in Cameroon as well as with the new HTLV-3 Pyl43 strain (Calattini et al., 2005; Courgnaud et al., 2004; Meertens et al., 2002). These results, like those obtained with STLV-1, also suggest regional spread of these genetically related STLV-3 strains within not only NHPs, but also humans as well, thus complicating a determination of the exact primate origin of HTLV-3 infections. We also identified for the first time novel STLV-3 in *L. albigena* and *C. cephus* which were closely related to each other and formed a distinct monophyletic clade among the Central African PTLV-3s. The high nucleotide sequence identity (99 to 100%) between the STLV-3 *tax*-pX-LTR sequences from *C. cephus* and *L. albigena* suggests a recent cross-species transmission in these monkeys. In a previous study, we showed that the prevalence of STLV infections in *C. cephus* is very low (less than 1%) (Courgnaud et al., 2004) and that those infections are mainly due to STLV-1. Our present results confirm these observations and show that, in contrast, *L. albigena* monkeys are predominantly infected by STLV-3. The cross-species event is therefore most likely to have occurred from *L. albigena* to *C. cephus*. This is consistent with these STLV-3 sequences being obtained from animals living in the same restricted geographic area and with observation of poly-specific associations between these two species in various regions of Central Africa (Bermejo, 1996; Williamson and Usongo, 1995). Nonetheless, screening of larger numbers of *L. albigena* and *C. cephus* and NHPs sympatric to these two monkey species in Cameroon is required to verify this hypothesis.

Our phylogenetic results confirm that PTLV-3s are separated according to their geographical origin (East, West and West-Central Africa) (Calattini et al., 2005). However, the acquisition of new genomic data for Central African PTLV-3s allows us to propose the existence of additional subtypes. Indeed, Central African PTLV-3s can be further separated into three distinct lineages: STLV-3 from *C. agilis* together with STLV-3 from Cameroonian wild-caught *C. torquatus* and HTLV-3(Pyl43) form one lineage, STLV-3 from *L. albigena* together with STLV-3 from *C. cephus* form a second lineage, and STLV-3 from *C. nictitans* forms a third and highly divergent PTLV-3 lineage. In addition, the presence of closely related STLV-3 strains in the same host species occurring in distant regions of Cameroon is consistent with a long and independent virus–host species co-evolution.

Although we observed a broad range of antibody profiles in wild-caught primate bushmeat samples from Cameroon, including HTLV-1-like, HTLV-2-like, dual HTLV-1/2 and indeterminate patterns, we were only able to detect STLV-1 and STLV-3 sequences in these primate samples. Using generic primers capable of detecting all four PTLV groups, we exclusively found STLV-1 genotypes in blood samples with HTLV-1-like WB profiles, while STLV-3 and dual STLV-1 and STLV-3 infections were observed in blood samples with HTLV-2-like, HTLV-1/2-like, and indeterminate WB patterns. While these results are consistent with the WB profiles observed in STLV-1 and STLV-3 and HTLV-3 infection (Calattini et al., 2005; Courgnaud et al., 2004; Georges-Courbot et al., 1996; Goubau et al., 1994; Makuwa et al., 2004a,b; Meertens et al., 2002; Nerrienet et al., 2001; Takemura et al., 2002; Van Dooren et al., 2001a,b; Wolfe et al., 2005), such broad PTLV seroreactivity limits the diagnostic utility of these results. Until improved serologic assays become available which can accurately detect and discriminate infection with each of the four PTLV groups, more laborious genotyping methods will continue to be required to differentiate PTLV infection. These new serologic assays will facilitate large scale population-based screening to determine the prevalence of the novel HTLV-3 and HTLV-4 viruses in order to better understand their public health significance. Nonetheless, our finding of divergent STLV-3s in many primate species across Cameroon suggests that additional HTLV-3 infections may be present in this country. Our inability to identify STLV-2 infection in NHPs in Cameroon suggests this virus may be rare in this region of Africa.

Finally, the exploration of the relative levels of lineage divergence in our *tax/rex* phylogeny suggests that additional PTLV types should be defined. By reference to the novel HTLV-4(1863LE) strain as a proposed fourth PTLV group (Wolfe et al., 2005), STLV-1(MarB43) should constitute a lineage separate from PTLV-1s that we tentatively name PTLV-5. Similarly, the taxonomical status of the highly divergent STLV-3-like viruses from Cameroonian *C. nictitans* as well as STLV-2s from *P. paniscus* should be reconsidered as possible new PTLV groups. However, our results are based only on a short region within *tax/rex* and do not yet allow a formal classification of these PTLVs. Further analyses encompassing larger parts of the genome are required to ascertain the taxonomy of PTLVs. Interestingly, a similar comparative exploration of relationship patterns between STLV-1(MarB43) and other PTLV-1s and between HTLV-4 and PTLV-2 using a phylogeny inferred from *pol* sequences confirms our preliminary observation (data not shown).

Materials and methods

Nonhuman primate sampling and blood collection

One thousand two hundred ninety-seven whole-blood samples were collected from wild-caught NHPs in southern Cameroon (Fig. 1) between 2001 and 2003. We sampled 1283 animals as bushmeat, available at markets surrounding villages or logging concessions in southeastern ($n=944$) and south-western ($n=353$) Cameroon, as well as 14 pet animals from the same areas (Table 1). All primate samples were obtained with government approval from the Cameroonian Ministry of Fauna and Forestry. Bushmeat samples were obtained through a strategy specifically designed not to increase demand: women preparing and preserving the meat for subsequent sale and hunters already involved in the trade were asked for permission to sample blood and tissues from carcasses, which were then returned. For the bushmeat animals, whole blood was collected by cardiac puncture. Animals and bushmeat seized by the National Program Against Poaching were also sampled. For pet monkeys, blood was drawn by peripheral venipuncture after the animals were tranquilized with ketamine (10 mg/kg). Plasma

and cells were separated on site by Ficoll gradient centrifugation. All samples, including peripheral blood mononuclear cells (PBMCs), plasma and whole blood, were stored at −20 °C. Species were characterized on site by visual inspection according to the *Kingdon Field Guide to African Mammals* (Kingdon, 1997). Information provided by the owners indicated that most of the animals had died 12 to 72 h prior to sampling. Table 1 summarizes the number of samples collected from each primate species in the current study.

Serologic testing

Plasma and whole blood samples were tested for the presence of HTLV cross-reactive antibodies using commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions, the MUREX HTLV-1+2 test (Abbott Laboratories, Wiesbaden, Germany) using synthetic peptides and recombinant proteins representing immunodominant regions of the envelope and transmembrane regions of HTLV-1 and HTLV-2 as antigens and/or Vironostika HTLV-1+2 micro-ELISA system (Organon Teknika Corp, Durham, NC) which contains purified HTLV-1 and HTLV-2 viral lysates and a recombinant HTLV-1 p21 envelope antigen. Samples were considered as ELISA reactive when the Optical Density/Cut off (OD/CO) ratio was equal or superior to 1. Samples reactive in the ELISA assay were retested with a commercially available confirmatory test, INNOLIA HTLV-1/2 (Innogenetics, Ghent, Belgium), a line immunoassay which discriminates between HTLV-1 and HTLV-2 cross-reactive antibodies as previously described (Courgnaud et al., 2004). This test configuration includes HTLV-1 and HTLV-2 recombinant proteins and synthetic peptides that are applied as discrete lines on a nylon strip. The antigenicity exhibited by these proteins and peptides is either common to HTLV-1 and HTLV-2 or specific to one of these two viruses allowing confirmation and discrimination in a single assay. Two Gag (p19 and p24) and two Env (gp46 and gp21) bands are included as non-type-specific antigens, which are used to confirm the presence of antibodies against HTLV-1 and HTLV-2. The type-specific antigens for HTLV-1 (Gag p19 and Env gp46) and HTLV-2 (Env gp46) are then used to differentiate between HTLV-1 and HTLV-2 infections. In addition to these HTLV antigens, control lines are present on each strip: one sample addition line (3+) containing anti-human immunoglobulin (Ig) and two test performance lines (1+and +/−) containing human IgG. All assays were performed and interpreted according to the manufacturer's instructions.

DNA extraction and PCR amplification

DNA was extracted from whole blood using Qiagen DNA extraction kits (Qiagen, Courtaboeuf, France). DNA integrity and primate species identity were confirmed by amplification of glucose-6-phosphate dehydrogenase gene (G6PDH) and/or mitochondrial (mt) 12sRNA gene as described (Peeters et al., 2002; van der Kuyl et al., 2000). To confirm the presence of PTLVs in samples with HTLV cross-reactive antibodies, a previously described diagnostic *tax-rex* PCR (220-bp) allowing generic as well as type-specific detection of PTLVs was done (Vandamme et al., 1997). The generic PCR proved to be highly sensitive in detecting all PTLV groups, and the discriminatory PCRs had high sensitivities and specificities to discriminate between PTLV-1, PTLV-2 and PTLV-3. These different nested PCR protocols have been previously described to detect one to five infected cells with a DNA input from 10^5 cells (Vandamme et al., 1997). In addition to the *tax-rex* fragment, we amplified for each STLV-1 and/or STLV-3 positive sample per monkey species (except for the agile mangabeys (*C. agilis*) an additional genomic fragment to confirm the phylogenetic clustering observed in the *tax-rex* region. We amplified a 450-bp LTR fragment for STLV-1 using a semi-nested PCRs with 8255not and LTRU5E primers for the first round and 8255not and 420LTR primers (Mahieux et al., 1997; Vandamme et al., 1997) for the second round with the following cycling protocol: denaturation at 94 °C for 2 min, 38 cycles of denaturation at 94 °C for 30 s, hybridization at 55 °C for 30 s and extension at 72 °C for 30 s with a final extension at 72 °C for 7 min. Similarly, a 900-bp fragment spanning the *tax*-pX-LTR region was sequenced for at least one STLV-3 positive sample per monkey species per site. For STLV-3, semi-nested PCRs were performed using AV45 and pX-LTR as primers in the first round and AV42 and pX-LTR as primers (Courgnaud et al., 2004) for the second round with the following conditions: denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 20 s, hybridization at 50 °C for 30 s and extension at 68 °C for 1.15 min with a final extension at 68 °C for 5 min. PCRs for both rounds were performed using the long expand PCR kit (Roche, molecular biochemicals, Mannheim, Germany).

For all PCR experiments, the reagents were prepared in a dedicated room. PCR products were purified on 1% agarose-gel with a Q.BIOgene GENECLEAN®Turbo kit (MP Biochemichals) and direct sequencing of both strands using the BigDye terminator technology was performed on an ABI 3130*xl* Genetic Analyser. Sequences were then assembled using the software package Lasergene (DNASTAR, Inc., MAD).

Phylogenetic analyses

Newly derived partial STLV *tax/rex*, *tax*-pX-LTR and LTR nucleotide sequences were each aligned manually with reference sequences from GenBank as well as STLV sequences previously characterized in the laboratory using Clustal X v.1.8 (Thompson et al., 1997), with minor manual adjustments. Nucleotide sites that could not be unambiguously aligned were excluded from the analyses. Appropriate models of evolution were selected for each data set using Modeltest 3.7 (Posada and Crandall, 1998) and maximum likelihood phylogenies were reconstructed using PHYML (Guindon and Gascuel, 2003). The analyses were performed using a discrete gamma distribution to account for variable substitution rates among sites with four rate categories and the TN93 model of substitution (Tamura and Nei, 1993) for all analyses. Nucleotide frequencies, nucleotide changes rate and gamma distribution shape parameters were estimated from the data. The starting tree

was obtained using BIONJ. Five hundred bootstrap replications were performed to assess confidence in topologies. The levels of divergence between clades in the *tax/rex* phylogeny were explored using the treePAT module in the Treedyn program (Chevenet et al., 2006). In order to explore the separate status of the newly characterized PTLV-1 01CM1374 strain isolated from *C. cephus*, a four-cluster likelihood mapping (Strimmer and von Haeseler, 1997) was performed on a reduced PTLV-1 LTR sequence set using Tree-puzzle v5.2 (Schmidt et al., 2002). This method computes the likelihoods for the three fully resolved trees possible for four sequences and plots them as points inside an equilateral triangle. Well resolved topologies appear in the corners while points in the center correspond to the absence of clustering (star-like evolution).

Nucleotide sequence accession numbers

The new sequences have been deposited in Genbank under the following accession numbers: AM712666–AM712673 (*tax*-pXLTR from STLV 3), AM712674–AM712680 (LTR from STLV 1), AM746628–AM746673 (*tax* partial sequences from STLV-1 and STLV-3).

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Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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